Journal of Biological Sciences 10 (1): 31-36, 2010
ISSN 1727-3048
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One Minute of Hypoxia from Carbon Dioxide During Infancy in *Rattus norvegicus* Diminishes Inhibitory Responses to Aversive Stimuli and Reduces Numbers of Neurons in CA1 Hippocampus

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Abstract: The aim of this study was to discern if a single brief episode of hypoxia during infancy would result in long-term changes in hippocampal organization and behavior. In a split-litter design male and female rats were exposed to either 1 min of carbon dioxide or control conditions on either postnatal day 8 or 18. When tested as adults there were no statistically significant treatment effects for ambulation, defecation or rearing in the open field. However, the rats rendered anoxic by carbon dioxide (CO_2) on day 8 showed significantly less freezing (less immobility) in a context associated with shock and less conditioned taste aversion but more proficient acquisition of a maze. Weights of major organs did not differ significantly between treatments. Cytometry indicated that the anoxic rats contained 20% fewer neurons in the CA1 but not the CA2 regions of the hippocampus. In conclusion, these results replicate previous studies and suggest that even a single brief anoxia before weaning can produce long-term adaptive consequences.

Key words: Anoxia, hypoxia, fear conditioning, hippocampus, tissue weights, bait shyness, carbon dioxide, spatial memory, learning

INTRODUCTION

Many studies have demonstrated that anoxia or protracted hypoxia during early life results in brain injury or different microstructural patterns with subsequent neurological and behavioral deficits (Nyakas *et al*., 1996). Forty-eight hours of intermittent hypoxia designed to mimic episodic obstructive sleep apnoea resulted in apoptosis in the cortex and hippocampus (Gozal *et al*., 2001). Other experimenters (Curristin *et al*., 2002) have demonstrated chronic sublethal hypoxia can disrupt synaptic development. One of the brain regions most susceptible to acute anoxia is the hippocampal formation, primarily CA1 (Sommer's Sector) which receives collateral input from the zinc-rich CA2, CA3 regions of Ammon's horn as well as the dentate fascia (Friede, 1966). Structural changes during ontogenesis of this area contribute to adult deficits for memory and learning (Roskoden *et al*., 2002).

Given the expected pockets of carbon dioxide that can occur in contained breeding spaces for wild and domestic species we were interested in the effects of transient anoxia due to saturation or near-saturation with carbon dioxide. In addition to affecting kinetics of neuronic carbonic anhydrase activity (Wong *et al*., 2004) five, 3 sec intracarotid injections of medical grade carbon dioxide into adult rabbits resulted in shrunken, densely stained neurons within the hippocampal formation (Wilson and Boxer, 2002). This zinc-containing metalloenzyme exhibits a six-fold increase in activity in rats between 10 and 25 days of age (Friede, 1966). We decided to investigate the long term effects of an even briefer (1 min) single, exposure to carbon dioxide during the period when synaptic development and maturational-increasing carbonic anhydrase activity would be most rapid. Such subtle yet permanent alterations could affect the adaptation of the individual and if sufficient in numbers, the group.

Golanski *et al*. (2001) found that only one, 60 sec of exposure to 100% carbon dioxide (20 psi) before weaning produced significant sex-specific deficits in adult contextual fear conditioning. This form of learning is important because it allows mammals to avoid contexts in which noxious or harmful stimuli have occurred. The present experiment was designed to replicate the robust deficits in conditioned fear reported earlier and to discern if this single, 60 sec exposure to anoxia from carbon dioxide resulted in permanent changes in the numbers of neurons and glial cells within the CA1 hippocampal formation.

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MATERIALS AND METHODS

Animals: The experiments were completed in the year 2002. The litters of 8, 120 female albino Wistar rats obtained from Charles River (Quebec) were culled to 4 male and 4 female pups L^{-1} within 24 h of birth (total n = 32). In a split-litter design half the numbers of males and females per litter were exposed to CO_2 while the other rats were handled in a similar manner but not exposed. Four litters were treated on postnatal day 8 while the remaining 4 L were treated on postnatal day 18.

Exposures: All pups from the same litter were removed from their mothers on the treatment day and taken to the room in which rats are killed by carbon dioxide according to the guidelines of the Canadian Council For Animal Care. Two males and two females from the litter were placed in a container and given the CO_2 (20 psi) for 60 sec as reported previously (Golanski *et al*., 2001). They were immediately removed and allowed to recover, as defined by a return of the righting reflex. This usually required between 2 and 4 min.

The other rats from the litter were placed in another cage. Condition was identified by a single ear mark that was counterbalanced for condition in different litters. After weaning rats were maintained, three per cage of the same sex, within a 12:12 L.D. cycle in standard wire cages in rooms. Temperatures were maintained within 1 of 20°C.

Behavioral testing: At 90 days of age the rats were tested for 2 min in an open field (Persinger, 1969). The numbers of squares traversed, rearings and fecal boluses were recorded. About five days later the rats were trained for conditioned learning. On the first day each rat was placed in a foot shock chamber and allowed 3 min to habituate. Unscheduled foot shocks (0.5 mA, 1 sec) were delivered once every 120 sec (total = 3) for 8 min. Twenty-four hours later the rats were returned to the same context but not shocked. They were observed 8 min during which time the freezing behavior was measured. The presence (indicated by 1) or absence of movement (indicated by 0) every 8 sec was recorded (total scored = 60).

About one month later, the rats (equal numbers from each condition) were trained for conditioned taste aversion. After habituating to 5 days of water availability for 0.5 h day^{-1}, the rats were given water containing 10% sucrose which was followed by an intraperitoneal injection of 10 mL kg^{-1} of 0.15 M lithium chloride. After two more days of only water during the consumptive interval, the 10% sucrose was made available for two successive days.

About one month later, the rats were habituated to a 23 h food deprivation schedule and trained in an automated spatial maze. There were four arms in the maze and at the end of each arm was an operant lever. A press of the lever resulted in the delivery of a Noyes pellet. The rat was required to press the lever of each arm at least once before return to a previous arm resulted in reward. The numbers of completions of the maze per 30 min were recorded for 8 consecutive days. The data were analyzed as four blocks of the means of two successive daily trials.

Histology: After the completion of the behavioral tasks, 16 of the rats (equal numbers from each treatment, time and sex) were weighed and decapitated. The following organs were weighed: brain, pituitary, spleen, thyroids, pancreas, adrenals and kidneys. The brains were quickly removed, fixed in ethanol-formalin-acetic acid, processed, embedded in paraffin and sectioned at 10 μm.

A total of 12 coronal slides within the caudal-rostral boundaries of the hippocampus were stained with toluidine blue O. The numbers of neurons (visible nucleus) and nuclei of oligodendroglia and astroglial cells within two 400 X fields within a 6×6 grid in both the CA1 and CA2 fields were counted. The total numbers were divided by 0.0625 to obtain the numbers of cells mm^{-2}.

Statistical analysis: Three way analysis (control vs. anoxia; days 8 vs. 18; male vs. female) of variance were completed for numbers of squares traversed, fecal boluses and rearings in the open field, freezing behavior in the contextual fear setting and amount of water or sucrose consumed for conditioned taste aversion. Four way analysis of variance with one level repeated (couplets of days) were completed for the numbers of successful completions of the maze and response/reward ratios. Post hoc analyses were Tukey's set at $p<0.05$. All analysis including exploratory factor analyses and covariance were completed with SPSS software on a VAX 4000 computer. In order to discern the strength of the treatment effects eta values from the analyses were included. The effect size, the amount of variance in the dependent variable explained or accommodated by the treatments, can be obtained by taking the square of the eta value.

RESULTS

The results of three-way analyses of variance demonstrated no statistically significant differences between the time of treatment, the type of treatment or the interaction for the numbers of squares traversed in the open field, numbers of rearings, or numbers of fecal

Table 1: Means and Standard Errors of the Mean (SEM) for various measures for rats tested as adults after being exposed for 1 min to CO_2 on either postnatal day 8 or day 18 or simply handled (litter mate controls)

	Control		Anoxia	
Measure	8 day	18 day	8 day	18 day
Freezing	50±3[a]	55±2[a]	36±8[b]	50±3[a]
Sucrose consumption				
Pairing Day	19±2	20±2	20±2	19±4
Test Day 1	5±2	5±1	9±2	7±1
Test Day 2	7±2[a]	9±2	15±2[b]	9±1[a]
Maze completions				
Block 1	16±4	9±2[a]	22±3[b]	12±2
Block 2	30±4[a]	25±5[a]	47±8[b]	26±4[a]
Block 3	40±5[a]	36±5[a]	58±8[b]	31±3[a]
Block 4	41±5	40±6	52±7	33±2
Bar Press/S+				
Block 1	5.2±0.8	7.3±0.7	4.8±0.4	5.9±0.4
Block 2	2.5±0.4	3.4±0.9	2.0±0.2	2.6±0.3
Block 3	1.7±0.2	2.1±0.4[a]	1.6±0.1[b]	2.0±0.1
Block 4	1.6±0.1	1.7±0.1[a]	1.3±0.1	1.8±0.1
Freezing maze factor	-0.2±0.4	-0.5±0.2	1.0±0.4	-0.4±0.3

a vs. b, $p<0.05$, Data is expressed as Mean±SEM

boluses during the 2 min of observation. The means and standard deviations for these measures were 44.6 and 23.5, 7.8 and 4.4 and 0.5 and 0.8, respectively.

Three-way analysis of variance demonstrated that the rats exposed to the 1 min of carbon dioxide displayed significantly [$F_{(1,16)} = 9.72$; eta = 0.37] less immobility (fewer freezing responses or poorer conditioning) compared to the handled but not treated litter mate controls (Table 1). Litters disturbed on postnatal day 8 exhibited significantly [$F_{(1,16)} = 9.49$, $p<0.01$; eta = 0.38] less freezing than those disturbed on day 18 and males exhibited more immobility [$F_{(1,16)} = 12.98$, $p<0.01$] than females. The only significant interaction [$F_{(1,16)} = 8.85$, $p<0.01$] between sex and time of treatment was due to the greater immobility exhibited by males exposed to the anoxic condition at 18 days of age compared to the other three groups.

The means and standard errors of the mean for the amount (in cc) of sucrose consumed on the pairing day and on each of the two testing days for the four groups are shown in Table 1. Only the amount of sucrose consumed on the second testing day differed significantly between groups. Post hoc analysis indicated that the rats exposed to the CO_2 on day 8 consumed significantly more sucrose (less conditioned taste aversion or bate shyness) than did the other three groups.

The means and standard errors of the means for the numbers of successful completions of the maze for the anoxic and control conditions treated on either day 8 or 18 are shown in Table 1. Four way analysis of variance (all dfs = 1,16) indicated the rats disturbed on day 8 completed more trials ($F = 11.37$, $p<0.01$; $eta^2 = 41\%$) than those disturbed on day 18 and females completed more trials ($F = 8.59$, $p<0.01$; $eta^2 = 34\%$) than males. The interaction between time of treatment and condition was significant statistically ($F = 4.26$, $p<0.05$; $eta^2 = 21\%$). The rats that received the anoxic condition on postnatal day 8 learned the maze more proficiently, as defined by more successfully completed trials, then the other three groups. Although, all groups improved over time, there were no statistically significant interactions with any of the main effects (all dfs = (3, 48), Fs<1.76).

Table 2: Means and Standard Errors of the Mean (SEM) for the numbers of neurons and glial cells in the hippocampus exposed for 1 min to CO_2 on either postnatal day 8 or 18 and their litter mate controls

	Control		Anoxia	
Measure	8 day	18 day	8 day	18 day
Cell counts				
Neurons CA1	1285±92[a]	1212±53	1028±36[b]	1070±79[b]
Neurons CA2	661±85	652±48[a]	688±58	710±47
Glial CA1	472±26	436±10	389±20	457±32
Glial CA2	717±32	704±32	664±34	686±39
Factor 1	-0.5±0.7	-0.3±0.3	0.3±0.5	0.2±0.5
Factor 2	1.0±0.3[a]	0.3±0.4	-0.9±0.3[b]	0.2±0.4

a vs. b $p<0.05$. Data is expressed as Mean±SEM

An exploratory factor analysis with the following four variables (factor scores in parentheses): defecation in the open field (-0.60), the freezing score in the contextual fear setting (-0.78) and the numbers of successful maze completions for the first (0.90) and second (0.91) blocks resulted in a single factor (Eigen value = 2.42). The most relevant result of the three-way analysis of variance was the interaction between time and condition [$F_{(1,16)} = 4.78$, $p<0.05$]. Post hoc analysis indicated that the factor score for the rats rendered anoxic on day 8 was significantly higher compared to all other groups.

The anoxic male rats were significantly [$F = 6.60$, $p<0.05$] heavier (Mean = 587, SD = 19) than their litter mate controls (Mean = 541, SD = 34). However, this treatment difference was not apparent for the females. The means and standard deviations for the weights for the rats from the anoxic and control conditions were 303 and 25 and 313 and 21, respectively.

Three way analysis of variance showed that the anoxic rats showed significantly [$F_{(1,10)} = 8.36$, $p<0.01$; eta = 0.58] fewer neurons in CA1 (Table 2). As indicated in Fig. 1a-d, the fewer numbers of neurons was also associated with a greater scattering of cells. Except for the significantly different numbers of neurons [$F = 12.08$, $p<0.01$; eta = 0.73] and glial cells [$F = 5.33$, $p<0.05$; eta = 0.51] between the two areas of the hippocampus, there were no other statistically significant main effects or interactions for the numbers of glial cells or neurons in CA1 or CA2.

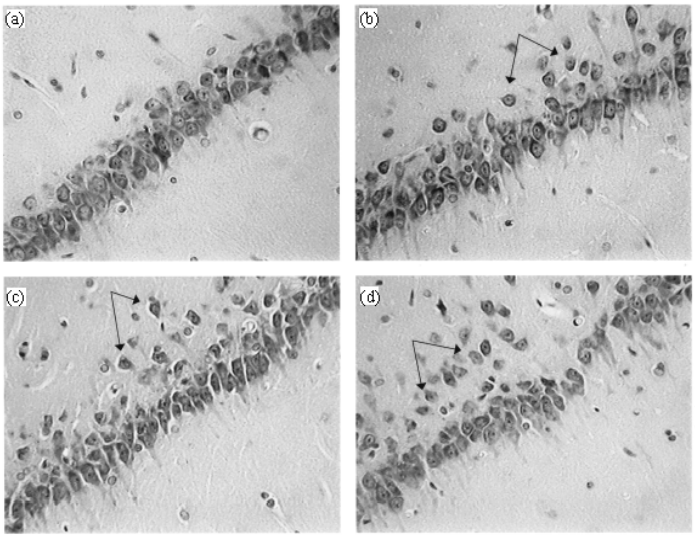

Fig. 1: Digitized photographs of soma of pyramidal cells within the CA1 section of the dorsal hippocampus (400 X) rats (b, c, d) from three different litters given carbon dioxide for only 1 min on postnatal day 8 and (a) a litter mate control

However, intensity of the freezing response was significantly correlated with the numbers of glial cells (r = 0.59) but not neurons (0.24) in CA1. The correlations between numbers of freezing responses and the numbers of neurons (0.32) and glial cells (0.26) within the CA2 region were not statistically significant. Factor analysis showed that the numbers of neurons in CA1 (-0.72) and CA2 (0.89) and glial cells in the latter (0.70) loaded on one factor (Eigen value = 1.87) while the numbers of glial cells in CA2 loaded (0.86) on the second factor (Eigen value = 1.13). The means and standard errors of the mean for the z-scores are shown in Table 2. Only the second factor showed a treatment effect [$F_{(3,14)}$ = 5.17, $p<0.01$; eta = 0.75] that was due according to post hoc tests to the lower scores (fewer glial cells in CA2) for the rats exposed to the carbon dioxide on day 8 compared to their litter mates.

Except for the obvious sex differences, that will not be reported here, there were no statistically significant differences between treatments, time of treatment or the interaction for the wet weights for spleen (M = 1.14 g; SD = 0.27 g), thyroids (M = 21 mg; SD = 6.6 mg), pancreas (M =.95 g; SD = 0.22 g), adrenals (M = 90.4 mg; SD = 16 mg), kidneys (M = 3.94 g; SD = 1.02 g), or brain (M = 2.02 g; SD = 0.46 g). Initial covariance for body weight did not reveal any recondite differences.

There were only seven statistically significant ($p<0.05$) correlations between the body weights and organ weights and the numbers of neurons and glial cells in CA1 and CA2 of the hippocampus. More numbers of neurons in CA1 were associated with heavier thyroids (r =0.60) and ovary weights (0.61) whereas fewer glial cells (-0.62) were associated with heavier testicle weights. More numbers of neurons within the CA2 area were associated with lighter body weights (-0.77), spleens (-0.62), thyroids (-0.79) and kidneys (-0.76).

DISCUSSION

The results of the present study demonstrated that only one minute of exposure to carbon dioxide can result in permanent changes in the numbers of cells within a portion of the hippocampus and behaviors frequently associated with that structure. These results partially replicated the reports by Golanski *et al.* (2001). They found that only the male rats exhibited attenuated freezing responses during contextual fear testing.

In addition, we found that rats rendered anoxic for 60 sec showed about a 20% reduction in numbers of neurons within the CA1 region of the hippocampal formation. Loss of neurons within the CA1 region of the hippocampus has been known for decades and can be precipitated by relatively minor episodes (Xia *et al.*, 2000) and lead to deficits in both learning and memory (Roskoden *et al.*, 2002).

Although, rats exposed to the carbon dioxide either postnatal day 8 or 18 showed fewer neurons compared to their litter mate controls in CA1 those exposed to the gas on day 8 also showed more irregular arrangements of neurons within the hippocampus. This group of rats were

also the ones that exhibited the significant attenuations of immobility to a context associated with a negative exteroceptive stimulus and a taste associated with a negative interoceptive stimulus but faster maze learning compared to the other three groups.

The absence of a simple correlation between numbers of neurons and scores for freezing responses suggests that the qualitative arrange or pattern of the neurons may be more important for the altered behaviors than simply the neuronal deficit. We did not measure neurons in other areas of the brain that may have been strongly correlated with the behaviors. We cannot also exclude the possibility that after a substantial number of neurons have been lost the relationship between structures and function may be qualitatively altered because of emergent properties which occur only when the more vulnerable subclasses of inhibitory neurons have been eliminated (Desjardins and Persinger, 1995; Persinger *et al*., 1997).

The rats exposed to the 1 min of hypoxia on either 8 or 18 days of age showed about 16% fewer pyramidal cells in CA1. Loss of hippocampal interneurons, which are predominantly inhibitory, is a common correlate of seizure activity which is frequently secondary to hypoxia (Sloviter, 1983). According to Wasterlain *et al*. (1990) there was no evidence of CA1 damage but clear indicators of cellular vulnerability within the inner layers of the dentate gyrus in rats in which seizures had been induced around 7 days of age.

However, status epilepticus during early life facilitates the genesis of an immature subset of granule cells within the dentate gyrus (Porter *et al*., 2004). Such change could alter synaptic organization of the hippocampal formation that is intimately involved with contextual learning. We cannot exclude the possibility that the marked behavioral changes we observed were due to interactions between the early hypoxic episode and the repeated stress of behavioral testing. Both behavioral stress (Xu *et al*., 1997) and inescapable (vs. escapable) shock (Shors et al, 1989) impair long term potentiation but facilitate long term depression in the hippocampus.

Although, many experimenters have reported the effects of hypoxia upon brain microstructure, the organism is a biological system and all other organs may be affected as well as the brain's subsequent interactions with these organs. Sarrat (1980) demonstrated reciprocal functioning between pancreatic islet beta cells and granule cells in the hippocampus. Lesions of either tissue disrupted the function of the other. From this perspective the reduced wet weights of the spleen, thyroid and kidneys associated with greater density of neurons in CA2 would suggest long-term modulations of the interactions between hippocampal function and the animal's immunological responsiveness (Bizere *et al*., 1985) and renal functions (Bradley *et al*., 1974).

The behavioral consequences of this single anoxic episode were specific to learning to avoid or to respond normally to novel or contextual stimuli associated with aversive stimuli. This may explain why the rats learned to acquire a complicated spatial maze so quickly. If there was diminished responsiveness to novelty or aversiveness, then acquisition of a complex spatial task associated with novel noises from the lever presses as well as the context would have been accelerated.

That this specific effect was not an artifact of hyperactivity was indicated by these rats' normal ambulatory and rearing behaviors within the open field test. In addition, the ratio of bar presses to rewards was not significantly greater than the other groups. If the rats had been hyperactive or perseverative one would have expected repeated bar presses subsequent to a press associated with a single reinforcement. Within a more ecological context diminished learning of aversive stimuli and enhanced learning rates could significantly alter the animal's response near human habitats.

CONCLUSION

This study shows that even 1 min of anoxia/hypoxia for inhalation of carbon dioxide before weaning produced long-term changes in behavior, an approximately 20% reduction of neuronal soma with mild organizational differences in the hippocampus. The treated rats showed less "fearfulness" within novel learning setting and following exposure to aversive stimuli compared to litter mate controls. Such brief periods may occur frequently within wild and domestic setting and may contribute to the behavioral variability in the population.

ACKNOWLEDGMENT

Thanks to Mathew Hunter and Viger Persinger for their technical assistance.

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